Supplemental Information

Supplemental Data

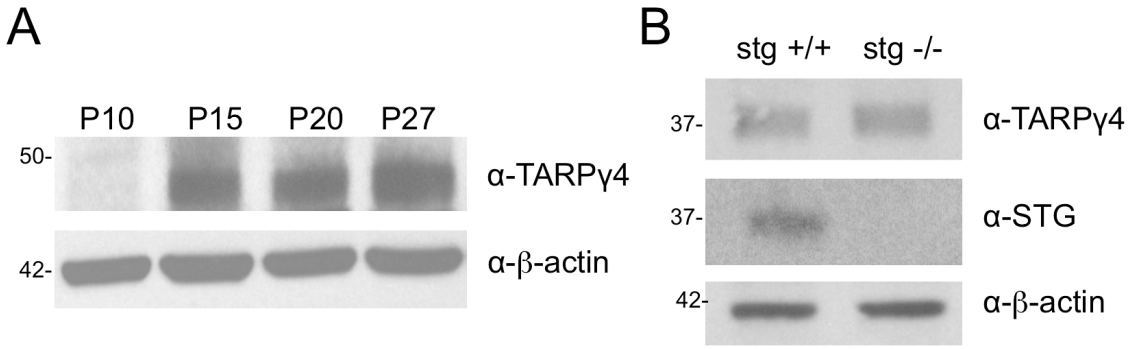

Fig. S1 (related to figure 1)- Expression of TARPγ4 in the LGN during development and in stargazer mice.

(A) Representative western blot for TARPγ4 total expression in wildtype mice over development. Notice the up-regulation of TARPγ4 after eye-opening and the maintenance of high levels up to P27. (B) TARPγ4 does not compensate for STG loss in the LGN of stargazer mice. Representative Western blot comparing TARPγ4 expression in stg +/+ and stg -/- P27 mice.

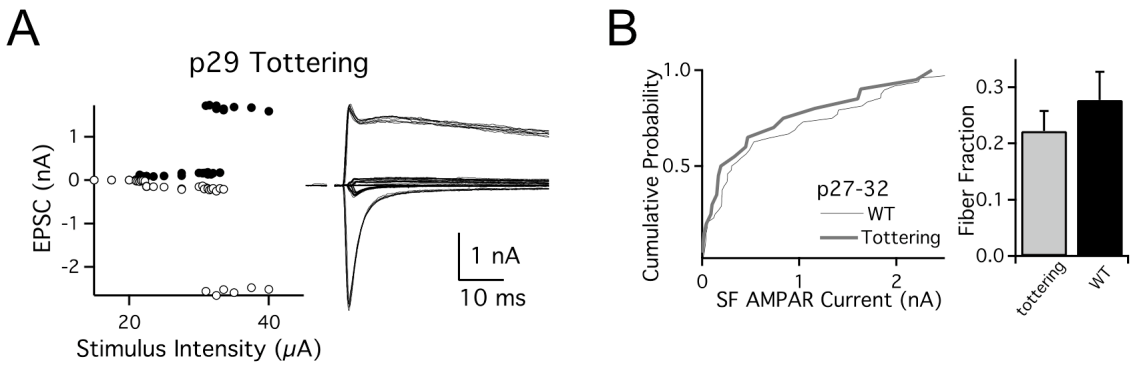

Figure S2 (related to Fig. 2) - Tottering mice exhibit normal synaptic refinement.

(A) Representative example of a synaptic recording from a P29 tottering mutant. (B) Comparison of cumulative probability plot of single fiber (SF) AMPAR current amplitude and fiber fraction of tottering to WT mice show no significant differences ($P>0.5$ for both SF and FF, Mann-Whitney, n=30-34).

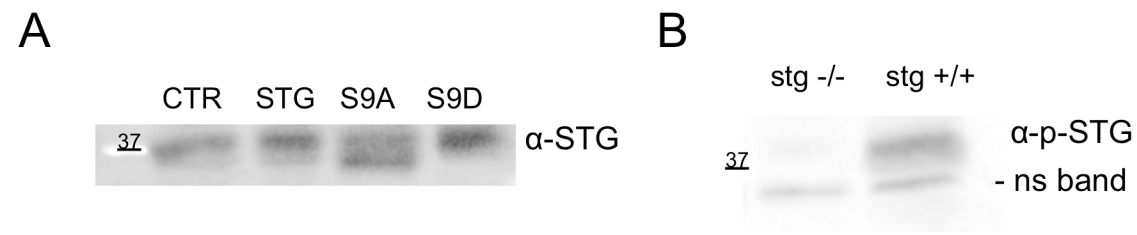

Fig. S3 (related to figure 3)- Monitoring STG phosphorylation state.
(A) Cortical neurons were transfected with WT STG or the phosphomutants S9A and S9D. Different phosphorylation states of STG can be monitored by western blot (as in (Sumioka et al. 2010; Tomita et al. 2005)). Over-expressing the phosphodead mutant - S9A - resulted in increased signal below 37 kDa whereas over-expression of the phosphomimetic S9D mutant resulted in increased STG band just above 37k Da. (B) Cortical lysates from stargazer (stg-/-) and wiltype (stg+/+) littermates were resolved by SDS-PAGE and probed with the phospho-serine 239/240 antibody. Please notice that phospho-STG specific band is above 37 kDa. (ns band – non-specific band)

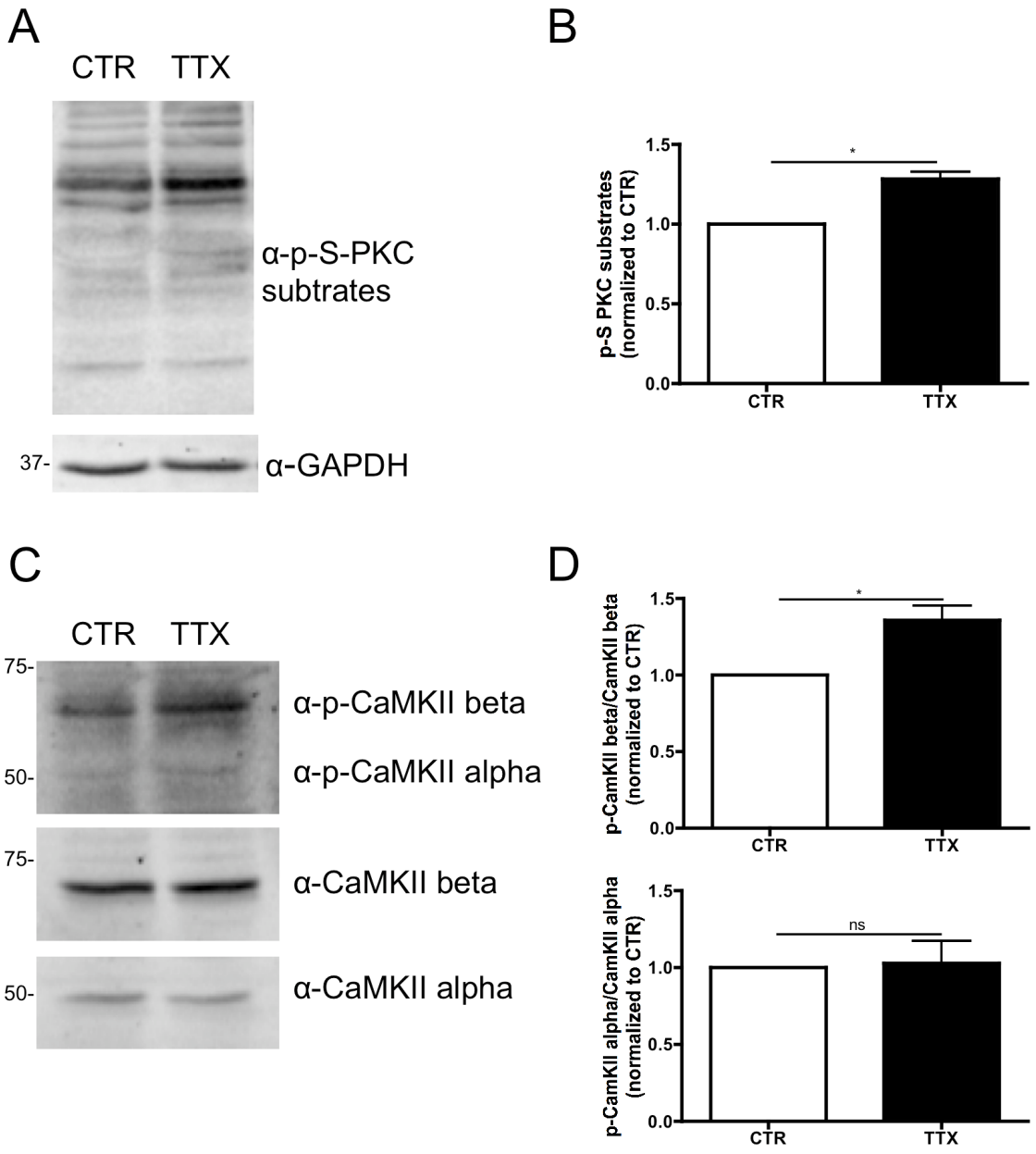

Fig. S4 (related to figure 7)- Signaling pathways in synaptic scaling.

(A) Cortical neurons were stimulated with TTX for 48h and the levels of different phospho-proteins were analyzed by western blot. (B) PKC activation was quantified by looking at the phosphorylation state of several PKC substrates. Prolonged activity blockade for 48h resulted in significant increase in the phosphorylation of PKC substrates (28.1 ± 5.0%). (C) CaMKII beta subunit is phosphorylated in synaptic scaling induced by TTX stimulation. Representative western blots of p-CaMKII, tCaMKII alpha and tCaMKII beta subunits and (D) quantification of the phosphorylation levels of CaMKII beta (top) and CaMKII alpha (bottom) isoforms. (N=3, t-test, * $P<0.05$).

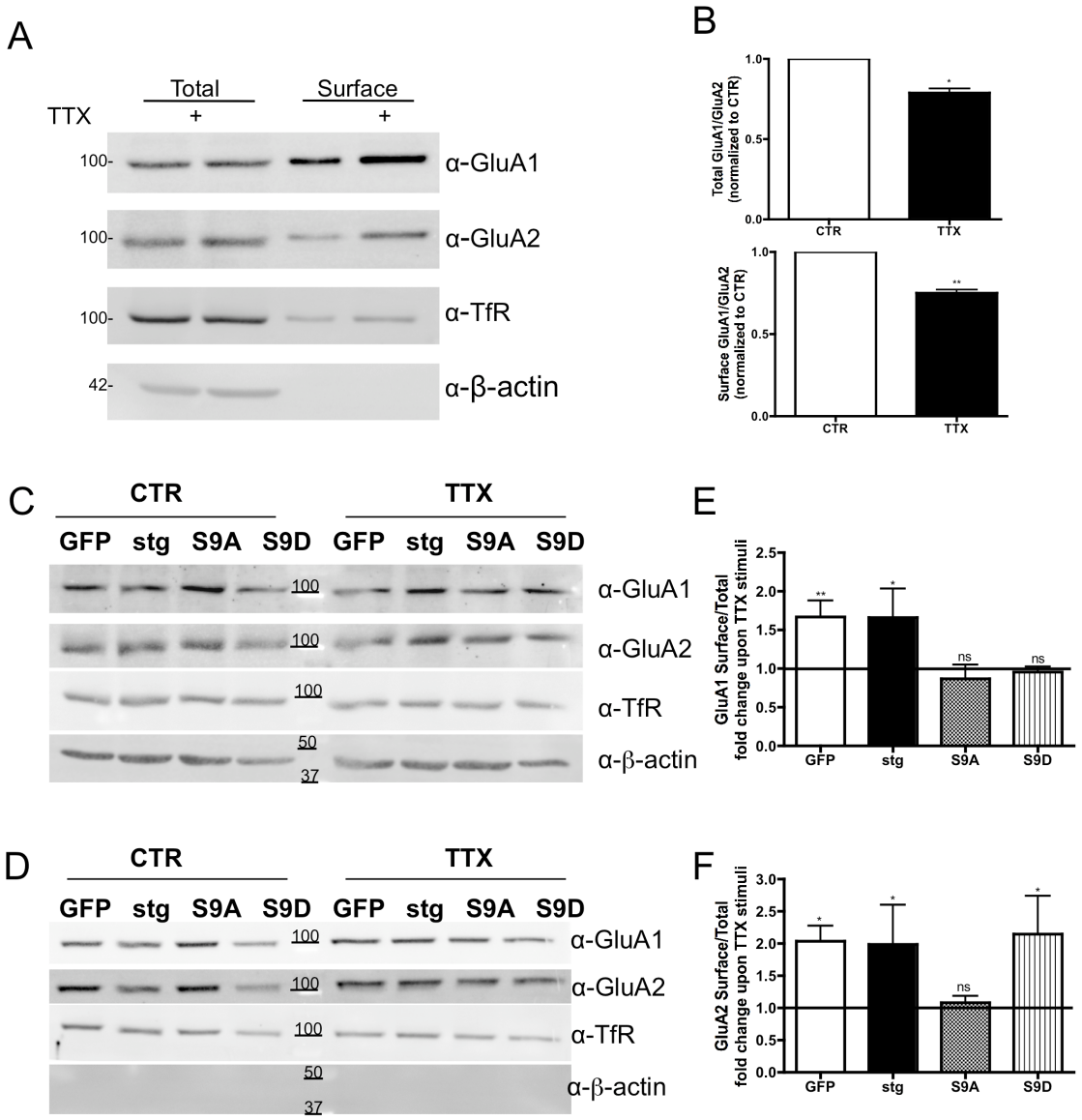

Fig. S5 (related to figure 7)- STG phosphorylation mediates synaptic scaling of both GluA1 and GluA2 AMPAR subunits.

(A) Representative western blots of total and surface fractions of DIV11 cortical neurons. (B) Chronic inactivity for 48h induces GluA2-contaning AMPAR accumulation at the surface of cortical neurons, resulting in a 25.7 ± 2.3% increase in GluA2/GluA1 surface ratio (*bottom graph)*. Total GluA2/GluA1 ratio was significantly decreased by 21.2 ± 2.8% (*top graph*). (** P<0.01, * P<0.05).
Representative Western blots of (C) input and (D) biotinylated fractions of DIV11 cortical neurons. Over-expression of stargazin S9A prevents both GluA1 and GluA2 increase in surface expression induced by TTX stimulation, whereas stargazin S9D mutant occludes GluA1 trafficking specifically. (E,F) Quantification of GluA1 (E) and GluA2 (F) fold change accumulation at the membrane of TTX-treated cortical neurons. (N=5 independent preparations, ** P<0.01, * P<0.05, significantly different from control, ANOVA, Bonferroni test).

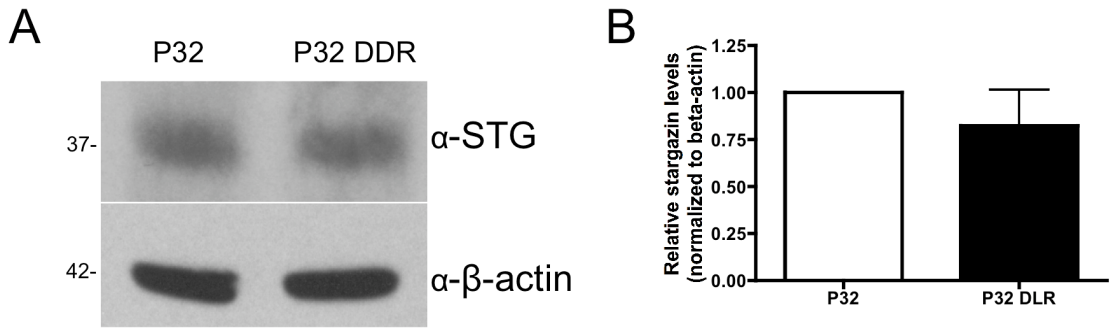

Fig. S6 – Dark-rearing mice after P25 does not affect STG expression in LGN.

(A) Representative western blots of P32 and P32 delayed-dark reared (DDR) mice, reared in the dark from P25 to P32.
(B) DDR does not affect STG expression in LGN (N=5, P=0.41, t-test)

Supplemental Experimental Procedures

Animals

Stargazer -/+ mice (B6C3Fe-a/a-Cacng2, Jackson Labs) were backcrossed with C57BL/6 to eliminate the retinal degeneration (RD) mutation present in the original mixed strain. Heterozygous mice were mated and the litters were genotyped for Cacng2 and Rd transcripts. Tottering mice (B6.D2 Cacna1atg/J) were purchased from Jackson labs and maintained on a C57BL/6 background. Genotypes were determined by PCR and confirmed by observation of an ataxic phenotype (for mutants).

Dark Rearing

Control animals for dark-reared experiments were raised in microisolator cages in standard animal facility conditions under a 12 h light/12 h dark cycle. For long-term experiments, mice were reared in complete darkness from P1 or earlier to avoid concerns that visual stimuli presented through closed eyelids (Akerman et al., 2002)

would affect results. Dark-reared pups and their mothers were placed in a light-tight container in which luminance was measured at <0.02 lux by photometer (Light ProbeMeter, Extech Instruments). Experimental dark-reared animals were transferred to the lab for slice preparation in an opaque box to minimize light exposure prior to sacrifice.

LGN slice preparation

Wildtype (stg +/+) and mutant (stg-/-) mice were bred from heterozygous parents. Mice (aged P11-32) were anesthetized using the inhalant isoflourane and decapitated. Acute LGN slices were prepared as described before (Chen and Regehr 2000).

Electrophysiology

Whole-cell voltage-clamp recordings of thalamic relay neurons from the dorsal LGN were performed as previously described (Chen, 2000). Cells located in the proximal 2/3 of the LGN relative to the optic tract (lateral and dorsal aspects of the dGN) were preferentially recorded from since they were more likely to have intact connections with the optic tract. Recording electrodes of 1.2-2.2 MΩ resistance were filled with a CsF-based internal solution of (in mM): CsF 35, CsCl 100, EGTA 10, HEPES 10, and pH 7.32 (with CsOH). 0.1 mM D600 (methoxyverapamil hydrochloride; Tocris, MO) was added to block voltage gated calcium channels. Slices were continuously perfused with isotonic saline and the $GABA_A$ receptor antagonist, 20 μM bicuculline (Tocris, MO) to inactivate local inhibitory circuits. A pair of glass filled saline electrodes was placed up to 1 mm away in the optic tract. Prior to recording from a given cell, the stimulating electrodes were moved to the location giving the largest postsynaptic response.

EPSC I-V analysis

Whole-cell voltage-clamp recordings of thalamic relay neurons from the dorsal LGN were performed. AMPAR EPSCs were recorded in the presence of CPP to block all NMDAR-mediated currents and spermine (Tocris, MO) was added to the internal solution as the internal polyamine. Voltage steps between -70 mV and +70 mV in 10 mV increments were repeated 5 times and AMPA-peak amplitudes were averaged and plotted against membrane potential. The rectification index was calculated by dividing the negative current at -60mV by the positive current value obtained at +40mV.

Lambda phosphatase assay

LGN tissue samples were prepared in TEEN buffer not supplemented with phosphatase inhibitors and 30μg of protein were incubated at 30ºC for 1h in the presence of lambda phosphatase (New England Biolabs, USA) according to the manufacturer protocol. The reaction was stopped by the addition of denaturing solution and the samples were resolved by SDS-PAGE.

Cortical neuron cultures (high and low-density)

Primary cultures of rat cortical neurons were prepared from the cortices of E18 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37 ºC; GIBCO Invitrogen), in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). Cortical cells were washed with HBSS 6 times. Cells were plated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid) in 6 well plates ($1.04x10^5$ cells/cm^2), coated with poly-D-lysine (0.1 mg/ml). After 2-4h the medium was replaced by fresh Neurobasal medium supplemented with SM1 (1:50), 0.5 mM glutamine and 0.12

mg/ml gentamycin.

For imaging purposes, low-density cortical cells were plated at a final density of 4 x 10^5 cells/dish on poly-D-lysine-coated coverslips, in 60 mm culture dishes, in neuronal plating medium. After 2-3 h, coverslips were flipped over an astroglial feeder layer. To prevent the overgrowth of the glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 days in vitro (DIV). Cultures were fed twice a week and maintained in Neurobasal medium supplemented with B27 supplement, in a humidified incubator of 5% CO2, at 37° C. To induce synaptic scaling, neurons were treated with 1 μM TTX for 48 h at DIV 8-9. At DIV10-11 the neurons were lysed with TEEN buffer (25 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl and 1% Triton X-100) supplemented with 50 mM sodium fluoride (NaF), 1.5 mM sodium orthovanadate (Na_3VO_4) and the cocktail of protease inhibitors.

DNA constructs

Stargazin wildtype plasmid was a kind gift from Dr. Veritty Letts (Jackson Laboratory, USA). Stargazin phosphomutants, S9A and S9D, were a kind gift of Dr. Susumu Tomita (University of Yale, USA). All small hairpin constructs were generated using the pLenti-Lox 3.7 backbone. The mock shRNA targets firefly luciferase and was described previously (Flavell et al., 2006). The following oligonucleotide was annealed with its complimentary sequence and inserted into the Hpa1/Xho1 sites of the pLenti-Lox vector: (shRNA#4) GAAGAACGAGGAAGTTATG. shRNA#-refractory stargazin (stgmt) was obtained by mutating two nucleotides in the shRNA recognition site: GAAAAACGAGGAGGTTATG. All constructs were verified by DNA sequencing.

Neuron transfection

Constructs were recombinantly expressed in primary cultures of cortical neurons using the calcium phosphate transfection protocol [adapted from (Jiang et al. 2004)]. This protocol is a low-efficiency transfection method that allows 20-50% of transfected neurons (Sun et al. 2013). Briefly, a $CaCl_2$ solution (2.5 M in 10 mM HEPES) was combined with plasmid DNA and then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, and 42 mM HEPES, pH 7.2). After 30min incubation the precipitated DNA was added to the coverslips and the cultures were incubated for 1.5 h in the presence of kynurenic acid (2 mM). Coverslips were then transferred back into the original astroglial plate and the plasmids were allowed to express during the indicated times.

Immunocytochemistry, culture imaging and quantitative fluorescence analysis

Neurons were fixed for 15 min in 4% sucrose/4%paraformaldehyde in PBS at room temperature, and permeabilized with PBS + 0.25% Triton X-100 for 5 min, at 4ºC. The neurons were then incubated in 10% BSA in PBS for 30 min at 37ºC to block nonspecific staining, and incubated with the indicated antibodies diluted in 3% BSA in PBS (2h, 37ºC or overnight, 4ºC). Imaging was performed on a Zeiss Axiovert 200 M microscope, using a 63x-1.4 NA oil objective. For quantification, sets of cells were cultured and stained simultaneously, and imaged using the same exact settings. Images were quantified using image analysis software (ImageJ). The region of interest was randomly selected and the dendritic length was measured using the MAP2 staining. For quantifying the GluA1 signal, fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. Surface GluA1 and stargazin digital images were thresholded such that recognizable clusters were included in the analysis. The Image J function, analyze particles, allowed us to calculate cluster intensity, number, and area of

the clusters for the selected region. The synaptic GluA1 and stargazin clusters were selected by their overlap with thresholded PSD95 signal. Measurements were performed in a minimum of three independent preparations, and at least 8 cells per condition were analyzed for each preparation. The analysis was performed blind to condition.

Supplemental References

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